DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

Version
January 1997

Compiled by

THE DIVISION OF AIDS

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and

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SPECIMEN PROCESSING

I. PRINCIPLE

The success of a protocol depends in part upon the adequate collection, preservation and retrieval of specimens. Guidelines for sample collection and storage need to anticipate the requirements of future studies which are yet to be designed and technological advances which are in the early stages of evolution. While this is not always possible, certain basic tenets exist. For example, all specimens should be collected and maintained using aseptic techniques. This includes the use of sterile tubes, pipette tips and reagents, and a work environment that is designed to prevent contamination of the sample.

II. SPECIMEN REQUIREMENTS

Consult specific protocol to determine the type and quantity of specimens required for each virologic assay.

III. REAGENTS

Dimethyl Sulfoxide (DMSO) - Store at room temperature. Use as long as it remains clear (4 - 5 years per Sigma Chemical).

Ice.

Cryoprotective Medium - Prepare enough medium for use and discard unused portion. Increase the following proportions (for 1 mL) as needed. Keep refrigerated.

0.4 mL RPMI
0.5 mL Fetal Bovine Serum
0.1 mL DMSO

For more reagents, see Qualitative PBMC Macrococulture Assay in this Manual.

Roche Specimen Wash Solution. Store at 4 - 8^{0}C and note manufacturer's outdate.

IV. EQUIPMENT AND SUPPLIES

Laminar flow hood (class 2 biosafety hood)
Gloves
Disposable lab coat
Microfuge capable of speeds > 10,000g

Centrifuge with horizontal rotor, capable of speeds up to 1800g and equipped with aerosol safe canisters
Sterile pipettes
Pipetting device
Sterile plugged pipette tips
Micropipettors at various volumes
Sterile cryopreservation vials
Sterile conical, cryopreservation vials
Sterile conical centrifuge tubes
Hemacytometer and microscope or flow cytometer for cell enumeration
-20^{0}C freezer
-70^{0}C freezer
Liquid nitrogen storage tank and boxes or canes
Surgical sponges
Betadine
60 cc syringe with 18 gauge needle
Sterile ACD solution

V. PROCEDURES

Primary Specimen Types

1. Whole Blood (BLD)

    a. Collection. Consult the specific protocol for vacutainer tube requirements.

    b. Processing.

        1) Acid Citrate Dextrose-A (ACD-A) or Heparin tubes should be processed within 4 - 6 hours of collection; consult the specific protocol for processing requirements. The tubes should be centrifuged at 1200g for 10 minutes to separate cells and plasma. The plasma is then removed avoiding the cell layer and centrifuged again at 1200g for 10 minutes to remove any contaminating cells and platelets. Plasma should then be aliquoted in sterile cryovials according to Virology Specimen Storage Recommendations in this manual or the protocol specific instructions, and stored at -70^{0}C. The PBMC are then separated as described in the Qualitative PBMC Macrococulture Assay in this manual. The PBMC can then be resuspended, counted and used for culture, stored as dry cell pellets or stored as viable cell suspensions.

        2) Citrate Cell Preparation Tubes (CCPT) should be processed within 4 - 6 hours; consult the specific protocol for processing requirements. The tubes should be centrifuged at room temperature in a horizontal rotor (swing-out

head) at a minimum of 1500g for a minimum of 20 minutes. Do not exceed 1800g, however, or some loss of cells or damage to the tube may result. After centrifugation, the plasma layer above the cells should be removed and centrifuged again at 1200g for 10 minutes to remove any contaminating PBMC and platelets. The plasma should then be aliquoted in sterile cryovials according to Virology Specimen Storage Recommendations in this manual, or the protocol specific instructions and stored at -70^0C. Add approximately 3.0 mL of PBS to the PBMC remaining in the CCPT tube, cap the tube, invert once, then remove the entire cell suspension and place into a sterile conical centrifuge tube. The cells are then washed once in calcium-free PBS and centrifuged at 400g for 10 minutes. The PBMC can then be resuspended, counted and used for culture, stored as dry cell pellets or stored as viable cell suspensions. If a CCPT tube is collected off site for next day delivery to a virology laboratory, the tube should be centrifuged within 3 hours to separate red cells and neutrophils from PBMC and plasma. The tube should then be inverted to mix the plasma and cells before placing the CCPT tube in the appropriate container for shipment. The receiving laboratory can then remove the entire layer above the gel and separate the PBMC and plasma with a 400g spin for 10 minutes. The plasma layer is removed and centrifuged at 1200g for 10 minutes to remove contaminating PBMC and platelets. The PBMC are then processed as above. This procedure of mixing the PBMC and plasma prior to shipping will result in a dilution of the plasma and should be noted. A dilution correction factor will then be used in calculating the RNA copy number. For information about training and how to purchase and receive the CCPT tubes, call Becton-Dickinson at (201) 847-4356.

2. Cord Blood (CRD)

a. Collection. After delivery of the infant, the cord is double clamped as close to the infant as possible. Cut between the clamps. After the infant is separated and while the placenta is <u>undelivered</u>, wipe the surface of the cord twice with a surgical sponge saturated in sterile saline in order to remove surface contamination with maternal blood or secretions. Prep a fairly large section of the cord, as multiple sticks may be required. The cord surface is then wiped with a betadine solution of at least 0.5% and allowed to dry for 30 seconds. Hold the cord down as low as possible to the remaining clamp. Puncture the umbilical VEIN as close to the clamp as possible, using a 60 cc syringe with an 18 gauge needle. This syringe must contain 3 mL of ACD solution prior to beginning the procedure. Aspirate venous blood until you meet resistance. Remove the syringe and raise the clamp above the puncture site. Restick the umbilical VEIN until the desired amount of blood is obtained, moving the clamp as you progress up the cord. (Some patients may be co-enrolled in other studies and may require large amounts of cord blood.)

Place 8 mL of cord blood in the appropriate CPT tubes for processing. Work must be done quickly as cord blood clots rapidly.

If the placenta is spontaneously delivered, place the placenta on a counter top and hold the cord below the surface of the counter and follow the above procedure.

Note: The collection of cord blood via the "dripping process" is not acceptable and not recommended due to increased risk of contamination between maternal and fetal blood.

b. Processing. See specific protocol for processing instructions.

3. Cerebrospinal Fluid (CSF)

a. Collection. 1-2 mL of CSF are collected in a sterile screw cap tube. Transport medium should not be used.

b. Processing. Freeze the specimen at -70^{0}C if a delay of greater than 6 hours is expected. If shipping is necessary, freeze at -70^{0}C and ship on dry ice. Vials should be labeled to indicate specific sample type.

4. Cervicovaginal Lavage (CVL)

a. Collection.

1) 10 mL of either 1x phosphate-buffered saline (PBS) or nonbacteriostatic normal saline (or normal saline) is drawn up through a plastic pipette into a 10 mL syringe. (The pipette is cut below the bulb and the remaining cath tip is inserted over the tip of a 10 mL syringe. Alternatively, a 14 gauge angiocath can be inserted over the tip of a 10 mL syringe.)

2) Introduce the pipette through the speculum into the vagina.

3) Squeeze the pipette to bathe the cervix (pipette should be directed towards the cervical os).

4) Allow the fluid to pool into the posterior fornix and aspirate into the same pipette.

5) Repeat this procedure 2-3 times with the same fluid; do not add any additional saline or PBS to the specimen.

6) Aspirate the fluid a final time and place into a cryovial for storage at -70^{0}C.

Note: Specimens should be transported to the laboratory in a timely fashion (within one hour). If this is not possible, place specimens on ice and refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

5. Endocervical Swab (CXS)

a. Collection

1) A Dacron swab is gently inserted 1 cm into the cervical os and rotated 360 degrees.

2) The swab is then placed into a sterile tube containing 2 mL of 1x PBS or non-bacteriostatic normal saline.

3) Rotate the swab 360 degrees against the inside of the tube to remove as much fluid as possible. Vortex the vial if this is possible; this procedure will aid in extracting the fluid from the swab.

4) Place the fluid in a cryovial.

5) Discard the Dacron swab.

Note: Specimens should be transported to the laboratory within one hour. If this is not possible, place specimen on ice or refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

6. Vaginal Swab (VAG)

a. Collection

1) A Dacron swab is inserted and rotated 360 degrees in all four quadrants of the vaginal vault.

2) The swab is then placed into a sterile tube containing 2 mL of 1x PBS or non-bacteriostatic normal saline.

3) Rotate the swab 360 degrees against the inside of the tube to remove as much fluid as possible. Vortex the vial if this is possible; this procedure will aid in extracting the fluid from the swab.

4) Place the fluid in a cryovial.

5) Discard the Dacron swab.

Note: Specimens should be transported to the laboratory within one hour. If this is not possible, place specimen on ice or refrigerate until transport.

b. Processing. The fluid may be aliquoted according to specific protocol instructions, but should be vortexed or agitated during this process to ensure equal concentrations in each aliquot. Freeze the sample as soon as possible at -70^{0}C or follow protocol specific instructions. Vials should be labeled to indicate specific sample type.

7. Other

Various tissues and body fluids may be required for protocol study. Consult specific protocols for collection and processing instructions. For example:

Brain biopsy (BRN)
Cervical biopsy (CVB)
Lymph node aspirate or biopsy (LYM)
Placental biopsy (PLC)
Saliva (SAL)
Semen (SEM)
Skin or lesion (SKN)
Spleen (SPL)
Tonsil aspirate or biopsy (TON)
Urine (URN)
Vaginal Secretions (VSC)

Derivative Specimen Types

1. Plasma (PLA)

a. CPT Tubes

Citrate Cell Preparation Tubes (CCPT) should be processed within 4 - 6 hours; consult the specific protocol for processing requirements. The tubes should be centrifuged at room

temperature in a horizontal rotor (swing-out head) at a minimum of 1500g for a minimum of 20 minutes. Do not exceed 1800g, however, or some loss of cells or damage to the tube may result. After centrifugation, the plasma layer above the cells should be removed and centrifuged again at 1200g for 10 minutes to remove any contaminating PBMC and platelets. The plasma should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C.

b. ACD or Heparin Tubes

Acid Citrate Dextrose (ACD) or Heparin tubes should be processed within 4 - 6 hours of collection; consult the specific protocol for processing requirements. The tubes should be centrifuged at 1200g for 10 minutes to separate cells and plasma. The plasma is then removed avoiding the cell layer and centrifuged again at 1200g for 10 minutes to remove any contaminating cells and platelets. Plasma should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C.

2. Serum (SER)

a. Processing. Allow the blood to clot, then centrifuge at 400-800g for 10 minutes. Serum should then be aliquoted in sterile cryovials according to the Virology Specimen Storage Recommendations or the protocol specific instructions and stored at -70^{0}C. If shipping the specimen, centrifuge as above and send at room temperature (20-24^{0}C). The serum should be aliquoted and frozen within 30 hours of collection.

3. Viable PBMC (CEL)

a. Collection. Obtain PBMC as described in Qualitative PBMC Macrococulture Assay in this manual.

b. Processing. Enumerate cells and resuspend PBMCs to a concentration of 2.5 x 10^6 to 10 x 10^6 PBMC/mL (keep on ice) with cold Cryoprotective Medium. The Cryoprotective Medium is added dropwise, with constant mixing, over 1-2 minutes. Dispense 1 mL aliquots of the cell suspension into cryovials. Place the cryovials in a small, insulated (Styrofoam), or slow-freeze container in a -70^{0}C freezer for 2-24 hours; then transfer to vapor phase liquid nitrogen (-135^{0}C) for storage.

4. Dry Cell Pellet for PCR (PEL)

a. Collection. Obtain PBMC as described in Qualitative PBMC Macrococulture Assay in this manual.

b. Processing. Aerosol resistant pipette tips should be used for this procedure. Enumerate the PBMC and adjust the sample with sterile PBS to achieve a concentration of 1-2 x 10^6 PBMCs/mL according to specific protocol instructions. The cell suspension should be dispensed into aliquots of 1 mL each in sterile cryovials according to Virology Specimen Storage Recommendations or the protocol specific instructions. Centrifuge for 3 minutes at the highest speed in a microfuge (typically >10,000 g). Aspirate the supernatant without disturbing the pellet. (Add 1 mL of Roche Specimen Wash Solution, vortex and centrifuge again for 3 minutes in a microfuge. Aspirate supernatant without disturbing pellet. If the pellet is not immediately extracted, it should be stored at -70^{0}C. Optional)

5. Whole Blood Pellet for DNA PCR (WBP)

a. Processing. Using aerosol resistant tips, add 0.5 mL of whole blood to a cryovial containing 1.0 mL of Roche Specimen Wash Solution. Cap the tube and mix by inversion several times. Incubate the sample for 5 minutes at 20-24^{0}C. Mix by inversion and incubate for 5 minutes more. Centrifuge for 3 minutes at the highest speed in a microfuge (typically > 10,000 g). Aspirate the supernatant without disturbing the pellet. Add 1.0 mL of the Roche Specimen Wash Solution to the pellet, vortex and re-centrifuge for 3 minutes as previously described. Repeat this wash step. Aspirate the supernatant without disturbing the pellet. If the pellet is not immediately extracted, it should be stored at -70^{0}C.

6. Supernatant from a Qualitative Culture (SUP)

a. Supernatant for p24 Antigen Testing - Supernatant is removed from a qualitative HIV coculture every 3-4 days as described in Qualitative PBMC Macrococulture Assay. Aliquot into a sterile cryovial and store at -20^{0}C or below until tested for p24 antigen.

b. Supernatant Collected at End of Positive Culture - When the qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, supernatant is harvested and aliquoted into sterile cryovials as described in Virology Specimen Storage Recommendations or specific protocol instructions. Store vials in vapor phase liquid nitrogen (-135^{0}C).

7. Previously Cultured Cells (PCC)

Aerosol resistant pipette tips should be used for this procedure. When a qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, the entire cell suspension is harvested and aliquoted into 1 mL samples according to Virology Specimen Storage Recommendations or specific protocol instructions. Store at -70^{0}C or below.

8. Cultured Cell Pellet (CCP)

Aerosol resistant pipette tips should be used for this procedure. When a qualitative HIV coculture meets the criteria for positivity as described in Qualitative PBMC Macrococulture Assay, the cells and supernatant are well mixed and removed to a sterile conical tube. Centrifuge the conical tube for 10 minutes at 1400 rpm. Remove and freeze the supernatant fluid if so directed by the protocol instructions. Resuspend the cell pellet in fresh culture medium or PBS and aliquot into 4 or more sterile, conical microcentrifuge tubes. Centrifuge tubes in a microfuge at maximum speed for 3 minutes. Remove supernatant and discard. Freeze pellets at -70^{0}C or below.

9. Supernatant from a Quantitative Culture (SPQ)

Supernatant for p24 Antigen Testing - On day 14 of the quantitative HIV microculture, supernatant is removed from each well and placed in a sterile cryovial until tested for p24 antigen. Store at -20^{0}C or below.

10. Presumed Low Passage Isolate (PLP)

When a quantitative HIV microculture is positive, pool the cells and medium into a conical centrifuge tube. Mix gently, then aliquot 1.5 mL of the cell suspension into the number of vials specified by the protocol. Store at -70^{0}C or below.

11. Ministock (STK)

    a. Collection. After the day 14 SPQ or PLP collection, the A1-B2 wells are fed with fresh donor cells and held to day 18. When a quantitative microculture meets the positivity criteria, supernatants and cells are harvested and stored. On day 18, remove supernatant fluids and cells from wells A1-B2 of the positive culture.

    b. Processing. Pool the cells and medium into a sterile conical centrifuge tube. Centrifuge at 400-800 g for 20 minutes at 20-24^{0}C. Aliquot the supernatant medium (STK) and store at -70^{0}C. The cells remaining in the centrifuge tube can be stored as a dry pellet (see CDP processing section) or a Presumed Low Passage Isolate (see PLP processing section).

12. Cultured Cells stored as a dry pellet (CDP)

    a. Collection. Cells are obtained from the ministock (STK) preparation above.

    b. Processing. Wash the cells by resuspending them in approximately 4 mL of sterile PBS. Aliquot into 2-4 sterile, conical microcentrifuge vials of 1 mL each. Centrifuge in a microfuge at maximum speed for 3 minutes at 20-24^{0}C. Aspirate the PBS; store the dry pellets at -70^{0}C.

13. Other

See individual protocol for special handling of the following specimens:

Cell pellet from cervical swab (CPE)
Cell pellet from vaginal swab (VPE)
Macrophage (MAC)
Supernatant from cervical swab (CSU)
Supernatant from vaginal swab (VSU)

VIROLOGY SPECIMEN STORAGE RECOMMENDATIONS (for ACTG laboratories)

The recommendations for adult and pediatric specimen storage have been developed for Adult ACTG and Pediatric ACTG laboratories. ACTG Investigators are strongly encouraged to use storage recommendations in all new ACTG study protocols whenever possible. Their use will save freezer space, which is at a premium in most labs, and will reduce technical error.

A. Specimens from Adult Patients

Serum (SER)	1.0 mL X 5 aliquots
Plasma (PLA)	1.5 mL X 6 aliquots
Cells DMSO (CEL) Viable uncultured cells	5×10^6/0.5 mL X 4 aliquots
Qualitative Culture Supernatants Isolated from positive qualitative cultures	1.0 mL X 4 aliquots used for additional isolates as needed
Presumed Low Passage isolate (PLP) Low passage isolate from positive quantitative culture	0.5 mL X 4 aliquots
Ministocks Quantitative Cultures (STK) Isolate from positive quantitative cultures	1.0 mL X 4 aliquots
Cultured Dry Cell Pellets (CDP) Non-viable cell pellet from positive quantitative culture	4 microcentrifuge tubes (uncounted)
PCR	Whole blood processed per kit requirements in individual laboratories. Storage of PCR samples should be one of the following: (WBP) 3 Dry Pellets from 0.5 mL blood (PEL) 2 or more Ficoll Pellets 1×10^6 cells

B. Specimens from Pediatric Patients

The suggested amounts are for optimal storage and/or processing. If less blood is obtained than the 4 mL, please refer to the Priorities List on the following page for guidance.

Serum (SER)	0.5 mL X 4 aliquots
Plasma (PLA)	0.5 mL X 2 aliquots minimum, 4 aliquots

Cells DMSO (CEL) Uncultured cells - viable	5×10^6/0.5 mL X 2 aliquots (freeze in 2 aliquots if cell count is low)
Qualitative Culture Supernatant (SUP) Isolate from positive qualitative culture	1.0 mL X 4 aliquots
Dry Cell Pellets (PEL) Non-viable cells	1 whole blood pellet 0.5 mL
Presumed Low Passage Isolates (PLP) Low passage isolate from positive quantitative culture	0.5 mL X 4 aliquots
Ministocks Quantitative Cultures (STK) Isolate from positive quantitative culture	1.0 mL X 4 aliquots
Cultured Dry Cell Pellets (CDP) Non-viable cell pellet from positive quantitative culture	4 microcentrifuge tubes

PCR Whole blood processed per kit requirements in individual laboratories. Storage of PCR samples should be one of the following:

For infants less than 18 months of age:

(WBP) 3 Dry pellets from 0.3 mL blood

For infants older than 18 months of age:

(WBP) 3 Dry pellets from 1.5 mL blood
(PEL) 3 Ficoll Pellets 1×10^6 cells

C. Proposed Priorities Listing

For volumes of blood 2 mL:

Plasma: Test and store remainder
Qualitative Culture
PCR
Cells in DMSO
Dry Cell Pellets

PROTOCOL VIROLOGISTS SHOULD PROVIDE PRIORITY INSTRUCTIONS WHEN INSUFFICIENT SAMPLING IS AVAILABLE TO ACCOMPLISH PROTOCOL REQUIREMENTS.